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PATENT- OG VAREMÆRKESTYRELSEN

A METHOD FOR DETECTION OF COLORECTAL CANCER IN HUMAN SAMPLES

Field of the Invention

The present invention relates to a method of diagnosing colorectal cancer in human samples using several novel protein markers. Differential expression pattern of these markers are indicative of a person having colorectal cancer and/or predictive of the stage of the disease in a colorectal cancer patient.

Background

Colorectal cancer is one of the world's most common cancers and the second leading cause of death due to cancer in the western world. Investigations of colorectal cancer show that most colorectal cancers develop from adenomatous polyps. The polyps are usually small and pre-neoplastic growths, that develop on the lining of the colon and can over time progress into colorectal cancer. Colorectal cancer occurs as a result of a sequence of mutations during a long period of time and these mutations mark the several different pathological stages of the disease. A model put forward by Fearon and Vogelstein describes colorectal cancer progression from normal epithelia to metastasis through the phases of dysplasia, early, intermediate and late adenoma and carcinoma.

A rare, inherited condition called familial polyposis (FAP) causes hundreds of polyps to form in the colon and rectum and unless this condition is treated, FAP is almost certain to lead to colorectal cancer. These individuals are therefore in a special need for a accurate screening test, where biopsies can be taken from a polyp during colonoscopy and analyzed for neoplastic changes.

Several mutations in oncogenes and tumor-suppressor genes have been identified in colorectal cancers and some of them have been associated with the phases of the disease mentioned above.

The risk factors for developing colorectal cancer seem to be age, diet, colon polyps, personal medical history, family medical history and inflammatory bowel disease (Ulcerative colitis and Crohn's disease).

Colorectal cancer incidences and mortality rates increase with age and sharply so after the age of 60. It is estimated that more than one-third of colorectal cancer deaths could be avoided if people over the age of 50 had regular screening tests, since over 90% of all cases occur in people 50 and older. This is due to the fact that colorectal cancer is one of the most preventable cancers, if it is detected at its early stages. If screening tests were performed on the risk groups for colorectal cancer, it could help to prevent deaths due to the disease by finding pre-cancerous polyps so they can be removed before they turn into cancer.

Studies have shown that women with a history of cancer of the ovary, uterus, or breast have a somewhat increased chance of developing colorectal cancer. The risk of developing colorectal cancer the second time seems to be evident as well. So these findings suggest that personal medical history seems to be relevant in terms of the assessment of risk for colorectal cancer. The same seems to be true for family medical history. First-degree relatives (parents, siblings, children) of a person who has had colorectal cancer are somewhat more likely to develop this type of cancer themselves. Ulcerative colitis is a chronic condition where the lining of the colon becomes inflamed and persons having this condition are considered at a greater risk of developing colorectal cancer than others.

The usual diagnostic methods for colorectal cancer are procedures such as: sigmoidoscopy and colonoscopy, that involve looking inside the rectum and the lower colon (sigmoidoscopy) or the entire colon (colonoscopy) and allowing for removal of polyps or other abnormal tissue for examination under a microscope. A polypectomy is the removal of polyp(s) during a sigmoidoscopy or colonoscopy, which is a procedure, often performed on individuals suffering from FAP and individuals with sporadic, recurrent colorectal polyps. Another way is to do X-rays of the large intestine, which is a technique that can reveal polyps or other changes in the intestine. A much less cumbersome method, but less indicative, is the fecal occult blood test (FOBT). It is a test used to check for hidden blood in the stool, as it has been observed that cancers or polyps can bleed, and FOBT is able to detect small amounts of bleeding in the stool.

The potential use of mass spectrometry as an aid for diagnosing cancer has been demonstrated in WO 01/25791 A2, disclosing protein markers from prostate cancer patients being differently expressed as compared to samples from healthy subjects or patients with benign prostate hyperplasia (BPH).

Several studies describe useful markers for the diagnosis of colorectal cancer. US 6,455,668 describes a screening method for identifying bioactive agents being capable of binding to a colorectal cancer modulating protein (BCMP). It further describes a method for screening drug candidates, wherein a gene expression profile is used including CJA8, or fragments thereof. The expression profile can further include markers selected from the group consisting of CZA8, BCX2, CBC2, CBC1, CBC3, CJA9, CGA7, BCN5, CQA1, BCN7, CQA2, CGA8, CAA7 and CAA9 (WO0055633). Another publication, US 2001/0044113, describes the use of PKC isozyme, in combination with more conventional cancer markers such as bcl-2, bax and c-myc, to detect changes in colonocyte gene expression associated with early stages of colon tumorigenesis by isolation of poly A+ RNA from feces. It should also be mentioned that the use of an undefined Defensin-polypeptide (Defensin-X) in diagnosing cancer is described in WO 99/11663.

There is, however, still unmet need for a simple diagnostic and/or prognostic test to provide an indication on whether or not an individual has colorectal cancer. It would also be of tremendous help to have a test giving indication on the status during surveillance of the disease.

Summary of the Invention

The present invention relates to a method of diagnosing colorectal cancer in a sample using novel protein markers. The markers have been identified, by assaying a number of tissue and serum samples from healthy individuals and persons diagnosed with colorectal cancer, by means of protein chip technology using mass spectrometry.

Differential expression pattern of these markers are indicative of a person having colorectal cancer and/or predictive of the stage of the disease in a colorectal cancer patient. The diagnosis is based on comparing an intensity value, obtained using the method, to a reference value.

Detailed description of the Invention

It is an object of preferred embodiments of the present invention to provide a method for diagnosing colorectal cancer in a sample from a mammal, the method comprising obtaining a sample from said mammal and assaying said sample by a quantitative detection assay, determining the intensity signal of at least one marker.

The at least one marker, such as two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, twenty-one, twenty-two or twenty-tree markers, can be selected from the group consisting of the polypeptides having apparent molecular weight of 2850 Da, 3570 Da (def 2), 3450 Da (def 1), 3480 Da (def 3), 4270 Da, 6850 Da, 9090 Da and 12000 Da, as well as 1945 Da, 2210, Da, 2230 Da, 2275 Da,3883 Da, 4300 Da, 4480 Da, 4500 Da, 5900 Da, 5906 Da, 3816 Da, 6436 Da, 13265 Da, 11133 Da and 13331 Da.

Thereafter, the method in a preferred embodiment comprises comparing said intensity signal(s) with reference value(s) and identifying whether the intensity signal of at least one marker from the sample is significantly different from a reference value.

In the present context, the term "diagnosing" includes determining whether a person has colorectal cancer as well as indicating the stage or prognosis of a cancer in a patient.

The term "colorectal cancer" relates to diseases such as colon cancer, familial adenomatous polyposis (FAP), rectal cancer and inflammatory bowel disease (IBD). It also relates to the non-invasive precancerous lesions such as adenomatous polyps.

In the present context, the term "phases of colorectal cancer" relates to the progressive stage of the disease. This diagnosis of the severity of colorectal cancer is most often based on pathological observations after surgery. This currently used diagnostic model describes colorectal cancer progression from normal epithelia to metastasis through the phases of dysplasia, adenoma (early, intermediate and late) and carcinoma.

In the present context, the term mammal refers to a primate, preferably a human.

In order to detect the presence of a gene product in a biological sample, one can measure either DNA/RNA or protein or both using quantitative detection assay(s). Such detection assay can be selected from the group consisting of immunoassay, kinetic/real-time PCR, 2D gel, protein array, gene array and other nano-technology methods.

In the present context, the term "immunoassay" refers to assays such as ELISA (Enzyme-Linked Immunosorbent Assay), RIA (Radioimmunoassay) and FIA (Fluoroimmunoassay), which are based on the ELISA sandwich concept of catching antibody and detection antibody with different specificity to the same molecule. The detection antibody is then labeled with an enzyme, fluorochrome or a radioactive substance or the like, to quantify the desired molecule (protein), and the sensitivity of the assay depends partially on the label of the detection antibody.

The term" 2D-Gel" (two-dimensional electrophoresis) relates in the present context to the electrophoresis technique where a protein extract is subjected to an electrophoresis in one dimension and then directly afterwards to a second electrophoresis in a second dimension. The conditions during the separate steps are different, in terms of time of separation, voltage, buffer and agents present during the separation.

In a preferred embodiment of the present invention mass spectometry is used to detect the protein markers. Furthermore the mass spectrometry method used is preferably a SELDI-TOF (Surface Enhanced Laser Desorption Ionization)-TOF (Time of Flight) technique, where the protein extract is bound to a protein chip. The chips have an active surface chemistry, which can be modified to retain proteins with certain properties. Thereby, proteins with different properties can be retained by different set of conditions and measured by MALDI-TOF or the like.

In the present context, the term "gene microarray" relates to low density nucleotide arrays, where nucleotide probes are attached or synthesized onto a surface and used as probes to retain nucleotides, mostly mRNA. This is usually referred to as transcription profiling, i.e. detection of the mRNA transcripts currently being used in a tissue at a certain time. Examples of such arrays are oligonucleotide arrays, where oligonucleotides are printed on glass slides and cDNA arrays, where cDNA (complementary DNA) is spotted on glass slide.

In a preferred embodiment of the present invention, the intensity signal detected in the quantitative detection assays is selected from the group consisting of fluorescense signal, mass spectrometry images, radioactivity, enzyme activity, and antibody detection.

The reference value can be calculated from a pool of samples from individuals with cancer and by comparison with a pool of samples from healthy individuals, a range for positive

and negative calls can be made. Another possibility is to set a reference value based on a pool of samples from various phases or stages of the cancer to determine the progression or a stage of the disease. It may even be desirable to set reference values for prognosis of the disease. The reference value can be calculated as a mean or a median value of each intensity signal value(s) calculated from data from one or many of the markers, wherein the negative values are made positive. The reference value could even be the area under the curve (AUC) of at least one of the protein markers.

In one embodiment of the present invention the reference value is indicative of the stage of colorectal cancer. This may be accomplished by collecting a number of samples from several patients and after the samples have been diagnosed by the stage of the disease, the samples from the same stage are assayed.

In the present context, the reference value can be based on data calculated from intensity signal value(s) of said marker(s) obtained from a sample without colorectal cancer from the same mammal. The reference value can also comprise data calculated from intensity signal value(s) of said marker(s) obtained from samples from normal and colorectal cancer tissue from the same mammal. Samples can furthermore be obtained from both a healthy control population and a population having said cancer which samples are used to determine the reference value. After the reference value is determined with a statistical significance, such as but not limited to p-values of levels below 0.1. By assaying a significant number of patients and healthy individuals, the specificity of the method can be determined, obtaining a specified sensitivity. Thereby, it can be determined whether a person is likely to have colorectal cancer or not with a predetermined specificity and/or a predetermined sensitivity.

In the present context the term "data" relates to any calculation made using the intensity signal(s) as data input. The intensity signal(s) may be fluorescence signal, mass spectrometry images, radioactivity, spectrometry values, etc. The data can be obtained using any kind of mathematical formula or algorithm.

Samples for setting the reference value will vary depending on the purpose of the assay. For diagnosis tissue samples may be taken from a "normal" tissue section and a cancer from the same individual, but reference samples may also be taken from healthy individuals in this context. It is also possible to collect blood samples from healthy individuals together with blood samples from individuals which are known to be suffering from colorectal cancer.

The prognosis of cancer patients is usually determined by the stage of the disease. The classification or the staging of the disease can be made using more than one model, but the most commonly used classification of colon cancer is based on the tumor morphology. This is the so called Dukes' classification (referring to the original classification described by Lockhardt-Mummery & Dukes in the 1930'ies) classifying the disease into three stages using the terms Dukes' A-C. Dukes A describes a cancer, where the cancer is limited to the

lining (mucosa or sub-mucosa) of the colon and has not penetrated the colon. At the Dukes' B stage, the cancer has penetrated the muscularis propria and invaded nearby organs. Dukes' C is characterized in that a regional metastasis of lymph nodes has occurred. Later, a commonly used stage "Dukes' D", referring to colorectal cancer with distant metastasis to organs like liver, lungs and brain was added to the classification. The 5 year survival prognosis for colorectal cancer is 80-90% at the Duke's A stage. Patients with Duke's B colorectal cancer have 60-70% 5-year survival rate whereas patients with Duke's C colorectal cancer are down to 20-30%. The 5 year survival rate for patients with Duke's D colorectal cancer is practically zero (Arends JW. et al.).

In a preferred embodiment of the present invention the reference value is indicative of the stage of colorectal cancer, wherein the stage is selected from the group consisting of Duke's A, Duke's B, Duke's C and Duke's D.

In the present context, the sample is a biological sample. The sample can be selected from the group consisting of blood, serum, plasma, feces, saliva, urine, a cell lysate, a tissue sample, a biopsy, a tissue lysate, a cell culture, semen, seminal plasma, seminal fluid and cerebrospinal fluid.

In a preferred embodiment of the present invention a protein extract is made from the biological sample containing the total protein content including membrane proteins, nuclear proteins, cytosolic proteins and blood/serum proteins. When the protein extract has been established, the protein concentration of the extract is made constant. In the present context the term constant refers to that the protein concentration of the sample to be analyzed should be standardized to a value being the same between different samples in order to be able to quantify the signal of the protein markers. Such standardization could be made using photometry, spectrometry and gel electrophoresis.

In a presently preferred embodiment of the present invention, the intensity signal for markers 2850 Da, 3570 Da (def 2), 3450 Da (def 1), 3480 Da (def 3), 4270 Da, and/or 6850 Da, is preferably raised, whereas the intensity signal for markers 9090 Da and/or 12000 Da is preferably decreased. These markers are preferably selected for evaluation of the presence of the disease from tissue samples or biopsies. Furthermore, for evaluation of the presence of the disease from blood samples, the intensity signal for 5900 Da, 3882 Da and/or 5906 Da, is preferably raised and the intensity signal for 3816 Da, 6436 Da, 13265 Da, 11133 Da Da and/or 13331 is preferably decreased.

In a most presently preferred embodiment of the present invention, the intensity signal for markers 1945 Da and 2210 Da is decreased and 5906 is raised. These markers are preferably selected for evaluation of the presence of the disease from blood samples.

In another presently preferred embodiment of the present invention, the intensity signal for markers 1945 Da, 2210 Da, 2230 Da, 2250 Da, 2275 Da, 4300 Da, 4480 Da and/or

4500 Da is decreased. These markers are preferably selected for evaluation of the presence of the disease from blood samples.

In further presently preferred embodiment of the present invention, the intensity signal for marker 5906 Da is raised. This marker is preferably selected for evaluation of the presence of the disease from blood samples.

Also in a presently preferred embodiment of the present invention, the intensity signal for marker 1945 Da is decreased. This marker is preferably selected for evaluation of the presence of the disease from blood samples.

Also in a presently preferred embodiment of the present invention, the intensity signal for marker 2210 Da is decreased. This marker is preferably selected for evaluation of the presence of the disease from blood samples.

Several mutations in oncogenes and tumor-suppressor genes have been identified in colorectal cancer. The majority of these genes are associated with certain phases of the disease. A mutation in the tumor-suppressor gene Adenomatous Polyposis Coli gene (APC), is considered to be a molecular "gatekeeper" for development of adenomas and it has been estimated that over 80% of all colorectal cancers have a somatic mutation in the APC gene. There are actually very few oncogenes, which have been shown to be involved with colorectal cancers apart from k-ras, but a small percentage of colorectal cancers show mutations in the myc, myb and neu oncogenes. A mutation in k-ras is considered to be an intermediate event in colorectal carcinogenesis advancing the disease from early adenoma to intermediate adenoma. Several other products of tumor-suppressor genes have also been associated with colorectal cancer, many of those genes are located on the long arm of chromosome 18. Allelic loss on 18q has been associated with the DCC gene (deleted in colorectal cancer), MADR2 gene (also known as JV18) and DPC4 gene (deleted in pancreatic cancer), the last two are players in the TGF-beta signaling pathway. It has been proposed that DCC, DPC4 and MADR2 play a role in the progression over to late adenoma (Gryfe R et al.).

One of the best known and studied tumor-suppressor genes, p53, is associated with driving the disease towards carcinoma. The product of the gene, which is located on chromosome 17, is a nuclear protein and has a function in cell cycle regulation, but a loss of heterozygocity on 17p has been demonstrated in over 70% of all colorectal cancers.

In a preferred embodiment of the present invention, the detection method using at least one of the novel protein markers for the detection of colorectal cancer could be supplemented with the detection of one or more protein markers selected from the group consisting of APC, k-ras, myc, myb, neu, DCC, DPC4, MADR2, p53, BCMP, CJA8, CZA8, BCX2, CBC2, CBC1, CBC3, CJA9, CGA7, BCN5, CQA1, BCN7, CQA2, CGA8, CAA7, CAA9, PKC isozyme, bcl-2, bax and c-myc.

Figure 13 presents the observed pattern of peptides in the region form 1900 to 2500 Da, the present inventors propose that the possible markers of values 1945, 2210, 2230, 2250 and 2275 Da. are somehow related. The pattern could indicate:

1) Fragmentation of a larger protein
2) Ligand binding peptides
3) Proteolytic processing of peptides
4) Translational / transcriptional regulation of peptides.

Figure legends

Figure 1.
Average intensity values of biomarkers of colorectal cancer. Tissue samples from 12 cancer patients including a normal tissue samples and cancer tissue sample from the same individual were homogenized and protein extracts were analyzed by mass-spectrometry using SAX2 chips and the SELDI-TOF technique. The figure shows the intensity levels of the markers selected based on highest sensitivity and specificity.

Figure 2.
Discriminating values calculated for 8 biomarkers. The average intensity value for each biomarker was calculated for normal and cancer tissue sample sets, after removing the highest and lowest values.The discriminating value for each biomarker was found by dividing the average intensities from each of the sample sets.

Figure 3.
Average intensity values of possible biomarkers in serum. Serum samples from 10 cancer patients and 10 healthy individuals were analyzed by mass-spectrometry using IMAC3 chips and the SELDI-TOF technique. The figure shows the intensity levels of the markers selected based on highest intensity.

Figure 4

Serum marker: 1945 Da.

Signal intensity	Cancer	Normal
middel	2,39339	24,94229
Max	8,899157	77,64356
Min	0,211373	2,690569

Threshold value: 8.9 (maximum value for cancer serum)
12 out of 78 normal serum samples fall below threshold, producing a specificity of 85 %.

Figure 5

SERUM marker 2210 Da

Signal intenisty	Cancer	Normal
middel	2,902108887	23,80824
Max	12,68954992	44,71738
Min	0,113351842	0,988566

Threshold value: 12.7 (maximum value for cancer serum)
18 out of 78 normal serum samples fall below threshold, producing a specificity of 77 %.

Figure 6

Serum marker 2230 Da

Signal intenisty	Cancer	Normal
mid	1,302903945	13,56049
max	5,682529669	31,203
min	0,012316878	0,637036

Threshold value: 5.6 (maximum value for cancer serum)
18 out of 78 normal serum samples fall below threshold, producing a specificity of 77 %.

Figure 7

Serum marker 2250 da

Signal intenisty	Cancer	Normal
mid	1,204193541	7,006661
max	3,640628662	20,46203
min	0,234108032	0,550792

Threshold value: 3.6 (maximum value for cancer serum)
22 out of 78 normal serum samples fall below threshold, producing a specificity of 72 %.

Figure 8

Serum marker 2275 da

Signal intenisty	Cancer	Normal
mid	0,821724872	4,189622
max	3,090245007	14,90973
min	0,125868733	0,245692

Threshold value: 3.1 (maximum value for cancer serum)
30 out of 78 cancer serum samples fall below threshold, producing a specificity of 62 %.

Figure 9

Serum marker 4300 da

Signal intenisty	Cancer	Normal
mid	0,358838372	2,662629
max	1,082232326	10,52571
min	0,029092626	0,225152

Threshold value: 1.1 (maximum value for cancer serum)
20 out of 78 cancer serum samples fall below threshold, producing a specificity of 74 %.

Figure 10

Serum marker 4475 da

Signal intenisty	Cancer	Normal
mid	0,828595247	3,363255
max	2,067939342	7,826388
min	0,035968835	0,900171

Threshold value: 2.1 (maximum value for cancer serum)
20 out of 78 cancer serum samples are below threshold, producing a specificity of 74%.

Figure 11

Serum marker 4500 da

Signal intenisty	Cancer	Normal
mid	0,821256006	3,360526
max	2,067939342	7,826388
min	0,035968835	0,889889

Threshold value: 2.1 (maximum value for cancer serum)
20 out of 78 cancer serum samples are below threshold, producing a specificity of 74 %.

Figure 12

Serum marker 5.9 Da.

Signal intenisty	Cancer	Normal
middel	5,088206618	1,413438
max	13,43115416	5,412548
min	0,638267678	0,182963

Threshold value: 5.4 (maximum value for normal serum)
49 out of 78 cancer serum samples fall below threshold, producing a specificity of 37 %.

Figure 13

Peptide pattern in the region from 1900 to 2500 Da.

Examples

Example 1 Identification of biomarkers for colorectal cancer by tissue investigations.

The aim of the study was to identify protein biomarkers indicative of colorectal cancer by comparison of normal and cancer tissue from colon and rectum.

Method

Sample preparation

Samples from 12 cancer patients were collected. Normal tissue samples and cancer tissue samples from the same colon were taken and frozen at -80°C. Prior to analysis the samples were taken out of the freezer and placed into homogenisation/Lysis buffer.

Lysis buffer:

100 mM TRIS, pH 8.0
9.5 M UREA
1% CHAPS.

The samples were homogenized in a Wheaton Overhead Stirrer for 2 minutes at speed step 2.

Analysis

Protein extracts were analyzed by mass-spectrometry using the SELDI-TOF technique.

SAX2 chips were pre-treated with 50 µl 100 mM TRIS pH 8.0 buffer.

10 µl homogenized sample + 60 µl TRIS pH 8.0 buffer were mixed and incubated on SAX2 Chip in a Bioprocessor for 30 minutes at room temperature. Thereafter spots were washed twice in 250 µl 100 mM TRIS pH 8.0 for 5 minutes.

2 times 0.5 µl Matrix (CHCA) was applied onto spot surface.

Instrument settings

Proteinchips were analyzed at Laser intensities of 190, 210, and 230, and the sensitivity level was set at 8.

Results

Putative biomarkers were identified by visual examination of the mass spectra from cancer and normal samples.

Table 1. Mass values of proteins showing increased expression in cancer tissue:

Laser Intensity		
190	**210**	**230**
2305 Da	2305 Da	2305 Da
-	2460 Da	2460 Da
-	2840 Da	-
2850 Da	2850 Da	2850 Da
2991 Da	-	-
3370 Da	3370 Da	-
3440 Da	3440 Da	-
3480 Da	3480 Da	-
-	4275 Da	4275 Da
-	-	6850 Da

Table 2. Mass values of proteins showing decreased expression in cancer tissue:

Laser Intensity		
190	**210**	**230**
1925 Da		
-		1940 Da
-		5000 Da
-		6190 Da
-	6375 Da	
-	6575 Da	
-		6590 Da
-		7570 Da
-		8410 Da
-		8700 Da
-		9090 Da
-		11670 Da
-		12000 Da

Possible biomarkers:

In order to the determine the specificity and sensitivity of the possible biomarkers all spectres were normalised based on total ion current.

Table 3. Specificity and sensitivity of protein biomarkers showing increased expression in cancer tissue:

Size (Da)	Specificity(%)	Sensitivity (%)
2300	83	66
2460	75	83
2850	100	92
2840	66	92
2990	75	50
3370	75	83
3450	83	83
3480	83	92
4270	92	92
6850	91	92

Table 4. Specificity and sensitivity of protein biomarkers showing decreased expression in cancer tissue:

Size (Da)	Specificity(%)	Sensitivity (%)
1920	75	50
1940	67	25
5000	50	50
6190	83	75
6375	67	100
6575	58	58
7590	83	50
8410	58	42
8700	66	58
9090	83	83
11670	83	50
12000	83	83

Possible multi-protein biomarker:

Based on values of sensitivity and specificity the most promising single protein biomarkers were selected:

Table 5. Protein biomarkers showing increased expression in cancer tissue:

Size (Da)	Specificity(%)	Sensitivity (%)	Identification
2850	100	92	nd
3370	75	83	Alfa-Defensin-2
3450	83	83	Alfa-Defensin-1
3480	83	92	Alfa Defensin-3
4270	92	92	nd
6850	92	92	nd

Table 6. Protein biomarkers showing decreased expression in cancer tissue:

Size (Da)	Specificity(%)	Sensitivity (%)	Identification
9090	83	83	nd
12000	83	83	nd

Conclusion

Eight promising single protein biomarkers were found using the SELDI-TOF mass-spectrometry technique and applying samples on protein-chips. Three of the markers have been fully identified as Alpha-Defensin 1, 2, and 3. A multi-protein biomarker based on a combination of one or more of the eight proteins shown above may prove to be a very effective way of screening for colorectal cancer.

Example 2 Identification of biomarkers for colorectal cancer in serum

The aim of the study was to identify protein biomarkers indicative of colorectal cancer by comparison of serum samples from normal and cancer patients.

Method

Sample preparation

Serum was isolated from blood of 10 patients diagnosed as having colorectal cancer and 10 healthy individuals.

Analysis

An IMAC3 chip was pre-treated with 2 times 5µl 100mM NiSO4, followed by wash with 5 µl MQ water and equilibration with 2 times 5 µl binding buffer.

Binding buffer:

100mM TRIS HCl, pH 7.5
500mM NaCl
0.1% Triton X-100

2 µl of each serum sample was diluted in 48 µl binding buffer of which 4 µl was applied to the protein chip surface. The chip was left on shaker at room temperature for 40 minutes. The sample was removed from the chip surface and each spot was washed with 3 times 5 µl washing buffer (PBS, pH 7.4, 700mM NaCl). Finally the chip was air-dried and 2 times 0.6 µl CHCA (100%) was applied to each spot.

Protein extracts were analyzed by mass-spectrometry using the SELDI-TOF technique.

Instrument settings

Protein-chips were analyzed at varying laser intensities and sensitivity levels to obtain optimal spectra.

Results

Sensitivity and specificity of putative serum markers:

Table 7. Protein biomarkers showing increased expression in serum samples of cancer patients:

Size (Da)	Specificity(%)	Sensitivity (%)	Identification
5905	70	70	nd
5899	70	70	nd
5928	70	70	nd
3882	60	60	nd

Table 8. Protein biomarkers showing decreased expression in serum samples of cancer patients:

Size (Da)	Specificity(%)	Sensitivity (%)	Identification
3816	60	60	nd
6435	60	60	nd
13265	60	60	nd
11132	50	50	nd
13331	50	50	nd

Conclusion

Eight possible single protein biomarkers were found using the SELDI-TOF mass-spectrometry technique and applying serum samples on protein-chips. None of the markers have been fully identified and annotated. A multi-protein biomarker based on a combination of one or more of the eight proteins shown above may prove to be a very effective way for diagnosis of colorectal cancer.

Example 3 Serum screening

Materials and method

Chip:

Serum samples were analysed on IMAC3 chip (Ciphergen).

Pre treatment:

Each spot is outlined with hydro pen.
5ul 100 mM NiSO4 is added, shake (150rpm) 1 min. Remove. Repeat once.
5ul MQ water is added, shake 1 min. Remove.
5ul Bind buf is added shake 1min. Remove.

Binding step:

Chip is placed in Bioprocessor.
50 µl binding buffer + 5µl serum is mixed in eppendorf tube, solution is loaded in bioprocessor.
Leave on shaker (250 rpm) for 40 min. Remove.

Washing step:

200 µl washing buffer is added. Shake (250 rpm) 5min. Remove. Repeat once.

Dry step:

Chips are removed from bioprocessor and left to air dry for 20 minutes.

Crystallisation step:

0.6 µl matrix solution is added to each spot. Air dry chip for 5 min.
Repeat once.

Analysis:

Chips are analysed on PBS II instrument (Ciphergen) at laser intensity 210 and detector sensitivity 4.

Results

Biomarker Wizard analysis

78 colon cancer serum and 78 normal serum samples were analysed as described above.

All spectra were pooled and normalised based on total ion current.

Possible biomarkers were identified by Biomarker Wizard (Ciphergen) analysis with the following parameter settings:

First pass:5, Min peak treshold: 0%, Cluster mass window: 0.3 %, Second pass: 5. Based on the results from the Biomarker Wizard 9 peptides showed promising marker characteristics.

Conclusion

Mass values of possible serum marker peptides:

Down-regulated in colon cancer serum:

1945, 2210, 2230, 2250, 2275, 4300, 4480, 4500 Da.

Up-regulated in colon cancer serum:

5906 Da.

Threshold values for possible serum markers

Optimal threshold values for the 9 serum markers were selected in order to determine maximum specificity of individual markers:

Marker (Da):	Specificity (%):
1945	85
2210	77
2230	77
2250	72
2275	62
4300	74
4480	74
4500	74
5906	37

Principal component analysis

Based on principal component analysis of a sample set of 38 cancer serum and 31 normal serum, it was shown that especially three markers were of high importance for discriminating between cancer and normal serum.

Especially important markers: 1945, 2210 and 5906 Da.

Note: Optimal conditions produces a mass accuracy of +/- 0.1%

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Claims

1. A method of diagnosing colorectal cancer in a sample from a mammal, the method comprising

obtaining a sample from said mammal

assaying said sample by a quantitative detection assay and determining the intensity signal of at least one marker selected from the group consisting of the polypeptides having apparent molecular weight of 2850 Da, 3570 Da (def 2), 3450 Da (def 1), 3480 Da (def 3), 4270 Da, 6850 Da, 9090 Da and 12000 Da as well as 1945 Da, 2210 Da, 2230 Da, 2275 Da, 3882 Da, 4300 Da, 4480 Da, 4500 Da, 5900 Da, 5906 Da, 3816 Da, 6436 Da, 13265 Da, 11133 Da and 13331 Da

comparing said intensity signal(s) with reference value(s)

identifying whether the intensity signal of at least one marker from the sample is significantly different from the reference value.

2. A method according to claim 1, wherein the reference value is/are intensity signal value(s) calculated from data of said marker(s) obtained from a sample without colorectal cancer from the same mammal.

3. A method according to claim 1 or 2, wherein the reference value is/are intensity signal value(s) calculated from data of said marker(s) obtained from samples from normal and/or colorectal cancer tissue.

4. A method according to any of the preceding claims, wherein the reference value is indicative of the stage of colorectal cancer.

5. A method according to any of the preceding claims, wherein the stage is selected from the group consisting of Duke's A, Duke's B, Duke's C and Duke's D.

6. A method according to any of the preceding claims, wherein the sample is selected form the group consisting of blood, serum, plasma, feces, saliva, urine, a cell lysate, a tissue sample, a biopsy, a tissue lysate, a cell culture, semen, seminal plasma, seminal fluid and cerebrospinal fluid.

7. A method according to any of the preceding claims, wherein a protein extract is made from said sample.

8. A method according to any of the preceding claims, wherein the protein concentration is made constant.

9. A method according to any of the preceding claims, wherein the quantitative detection assay is selected from the group consisting of immunoassay, kinetic/real-time PCR, 2D gel, protein array, gene array and other nano-technology methods.

10. A method according to any of the preceding claims, wherein the signal is selected from the group consisting of fluorescence signal, mass spectrometry images, radioactivity and enzyme activity.

11. A method according to any of the preceding claims comprising, supplementation with at least one marker selected from the group consisting of APC, k-ras, myc, myb, neu, DCC, DPC4, MADR2, p53, BCMP, CJA8, CZA8, BCX2, CBC2, CBC1, CBC3, CJA9, CGA7, BCN5, CQA1, BCN7, CQA2, CGA8, CAA7, CAA9, PKC isozyme, bcl-2, bax and c-myc.

12. A method according to any of the preceding claims, wherein the intensity signal for markers 2850 Da, 3570 Da (def 2), 3450 Da (def 1), 3480 Da (def 3), 4270 Da, and/or 6850 Da is raised

13. A method according to any of the preceding claims, wherein the intensity signal for markers 9090 Da and/or 12000 Da is decreased.

14. A method according to any of the preceding claims, wherein the intensity signal for 5900 Da, 3882 Da and/or 5906 Da is raised

15. A method according to any of the preceding claims, wherein the intensity signal for 3816 Da, 6436 Da, 13265 Da, 11133 Da Da and/or 13331 is decreased.

16. A method according to any of the preceding claims, wherein the mammal is a primate.

17. A method according to claim 16, wherein the primate is a human.

18. A method according to any of the preceding claims, wherein the intensity signal for markers 1945 Da and 2210 Da is decreased and 5906 Da is raised.

19. A method according to any of the preceding claims, wherein the intensity signal for markers 1945 Da, 2210 Da, 2230 Da, 2250 Da, 2275 Da, 4300 Da, 4480 Da and/or 4500 Da is decreased.

20. A method according to any of the preceding claims, wherein the intensity signal for marker 5906 Da is raised.

21. A method according to any of the preceding claims, wherein the intensity signal for marker 1945 Da is decreased.

28. A method according to any of the preceding claims, wherein the intensity signal for marker 2210 Da is decreased.

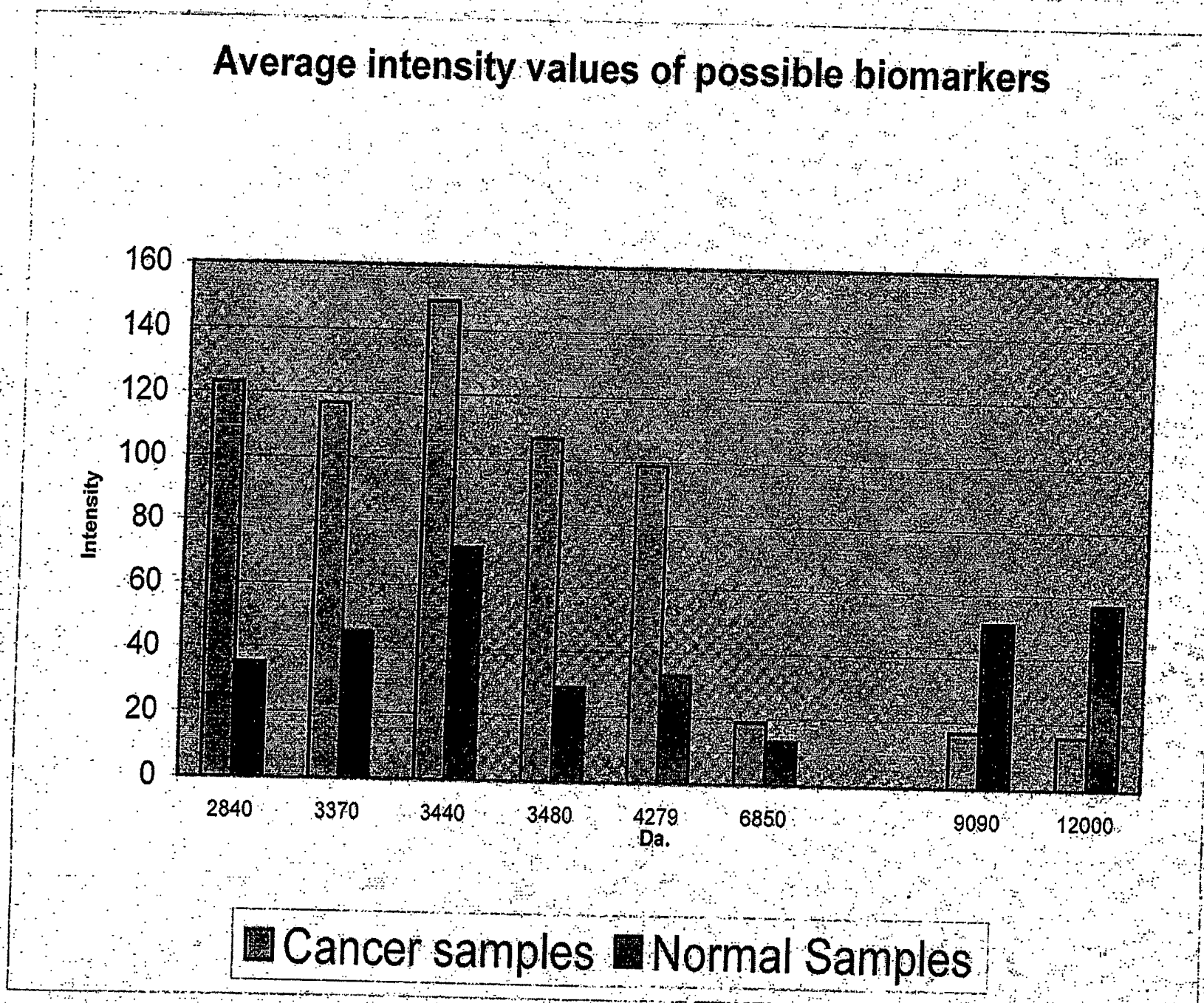
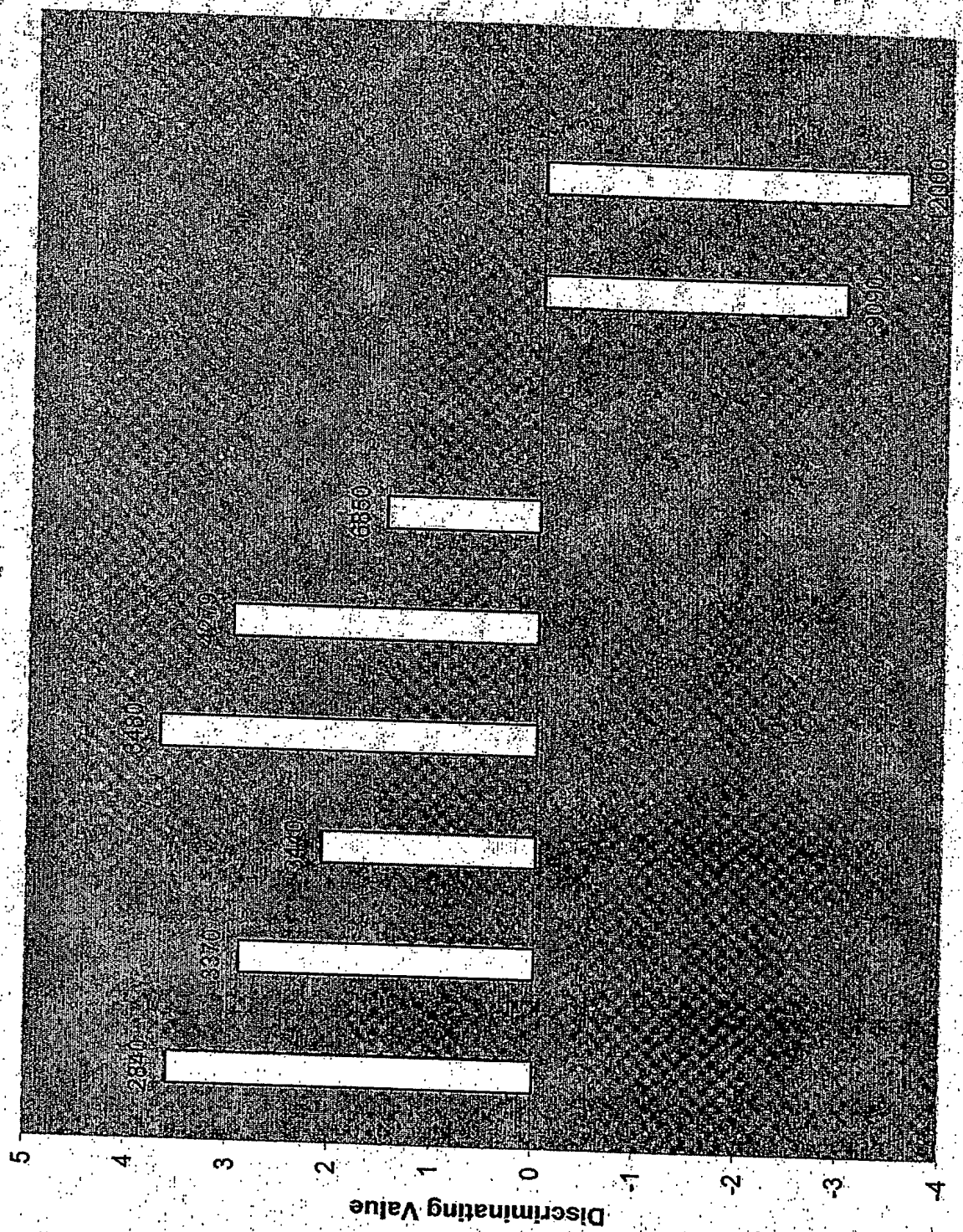


Fig. 1



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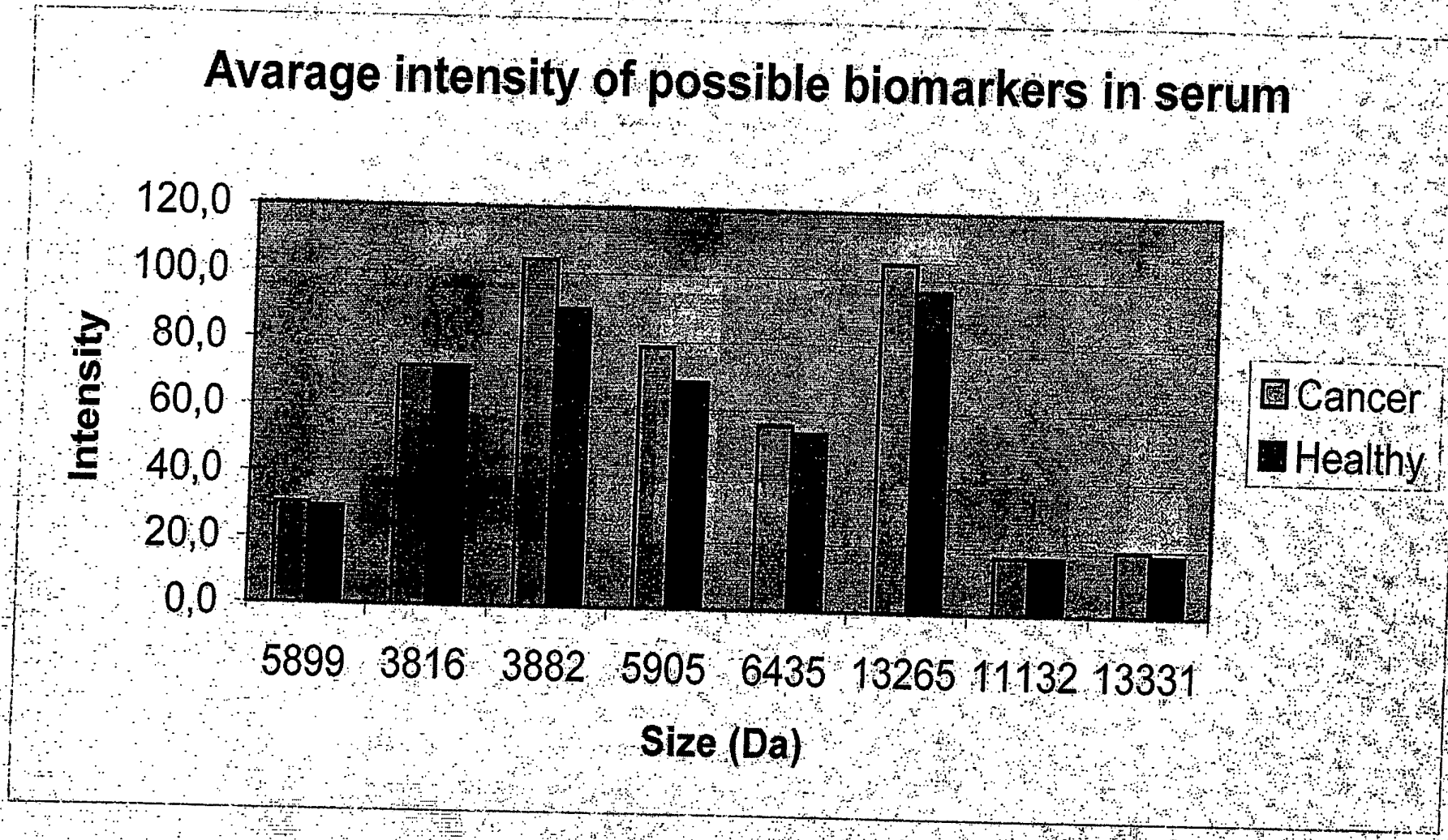

Fig.3

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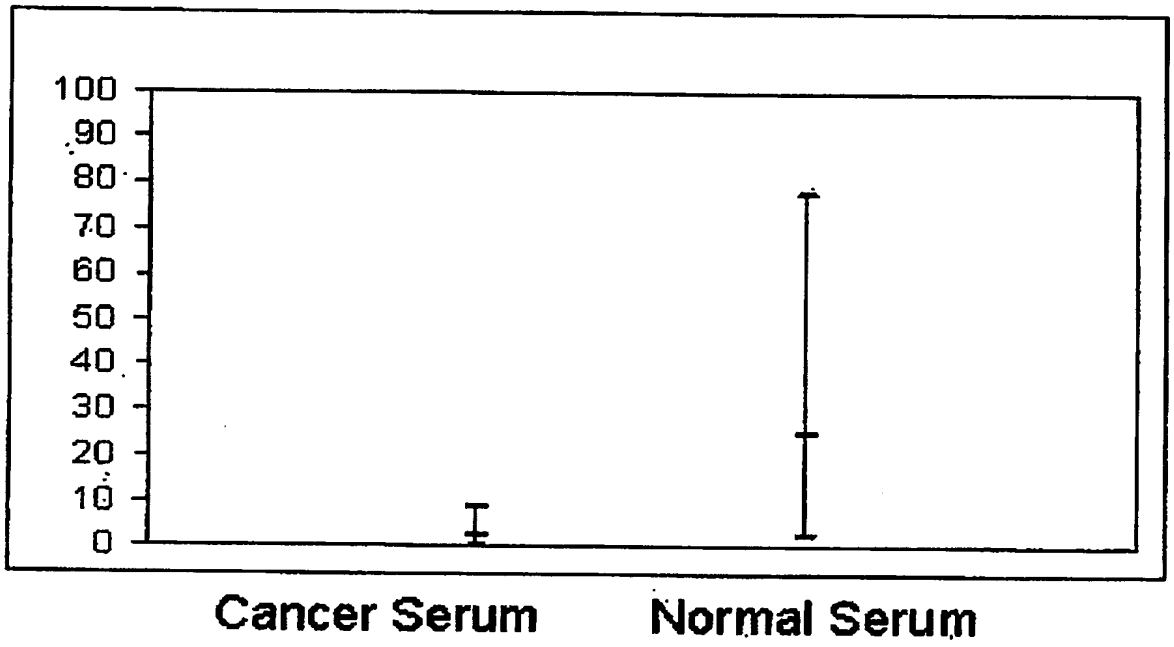

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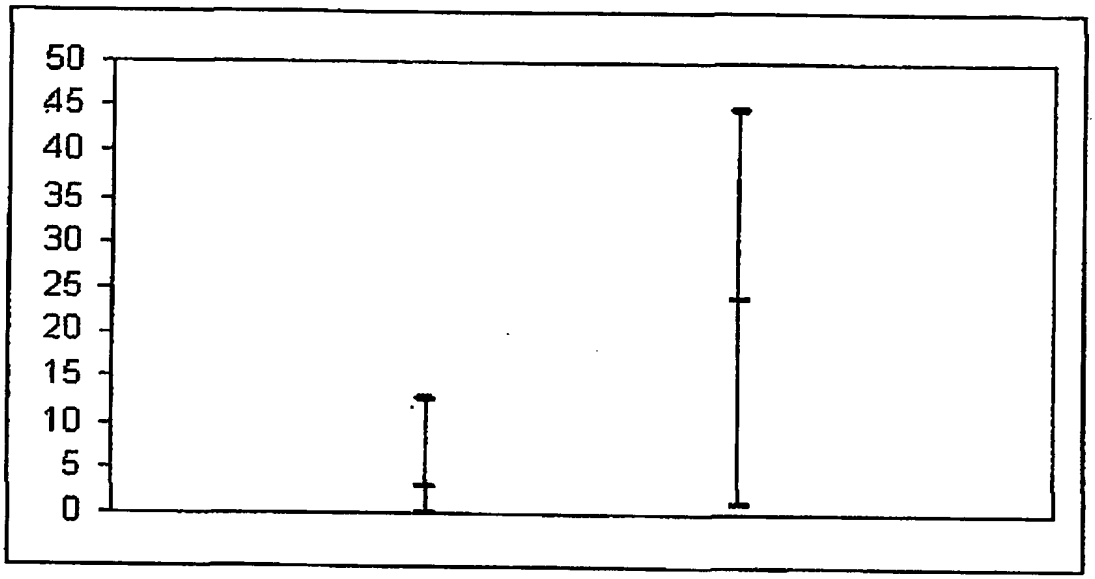

Fig. 5

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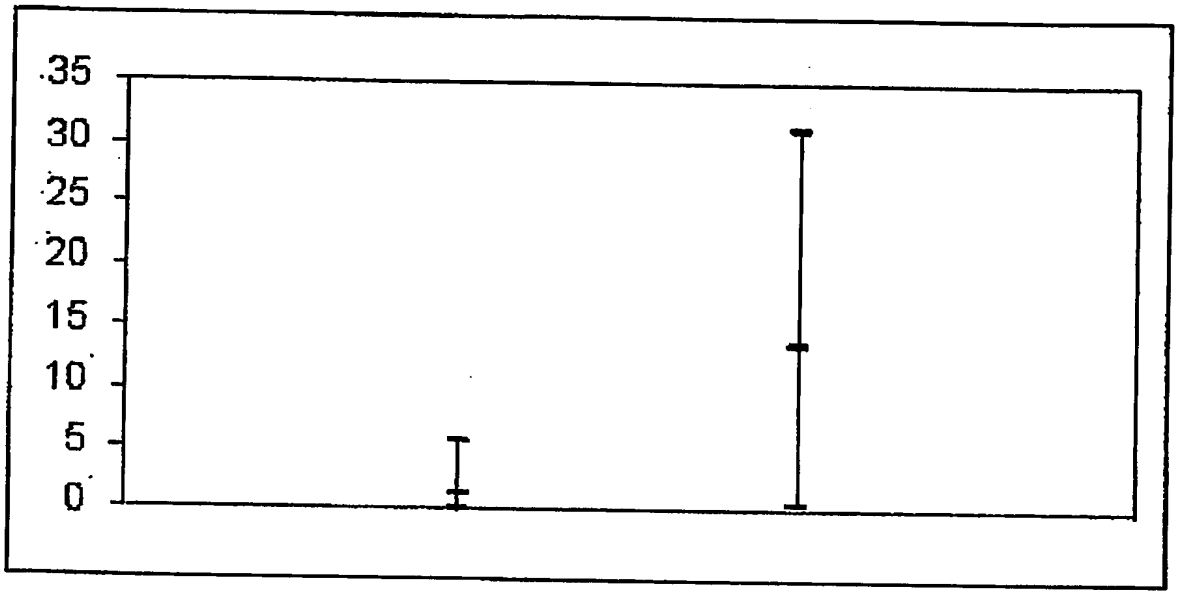

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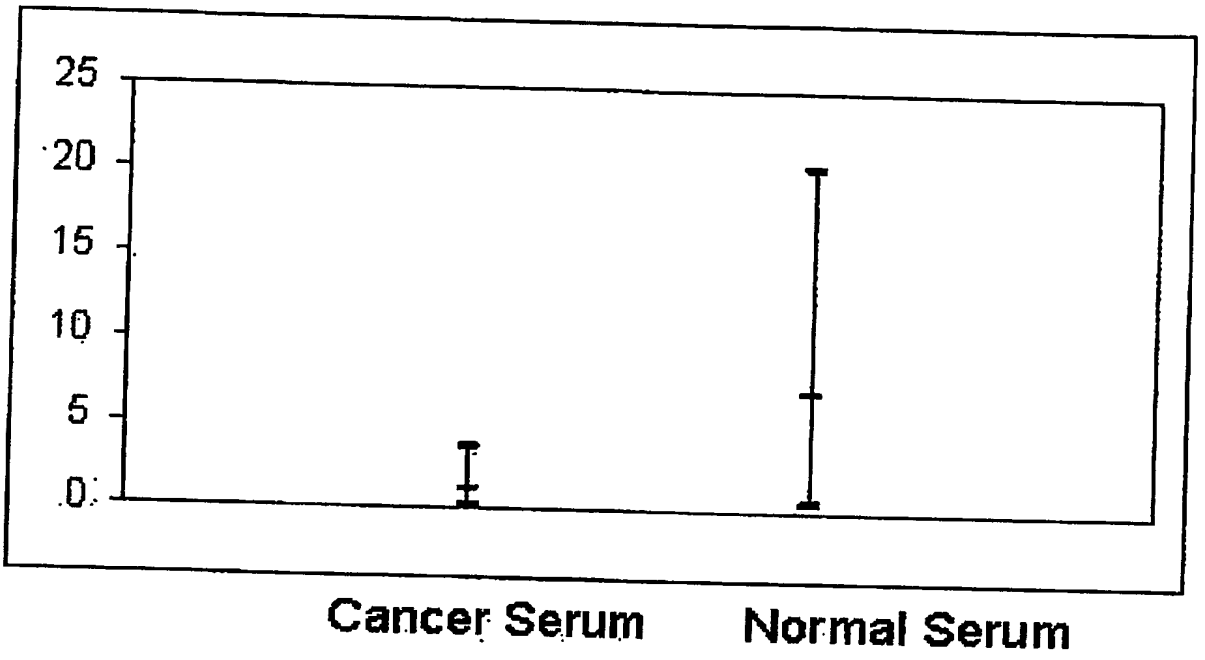

Fig. 7

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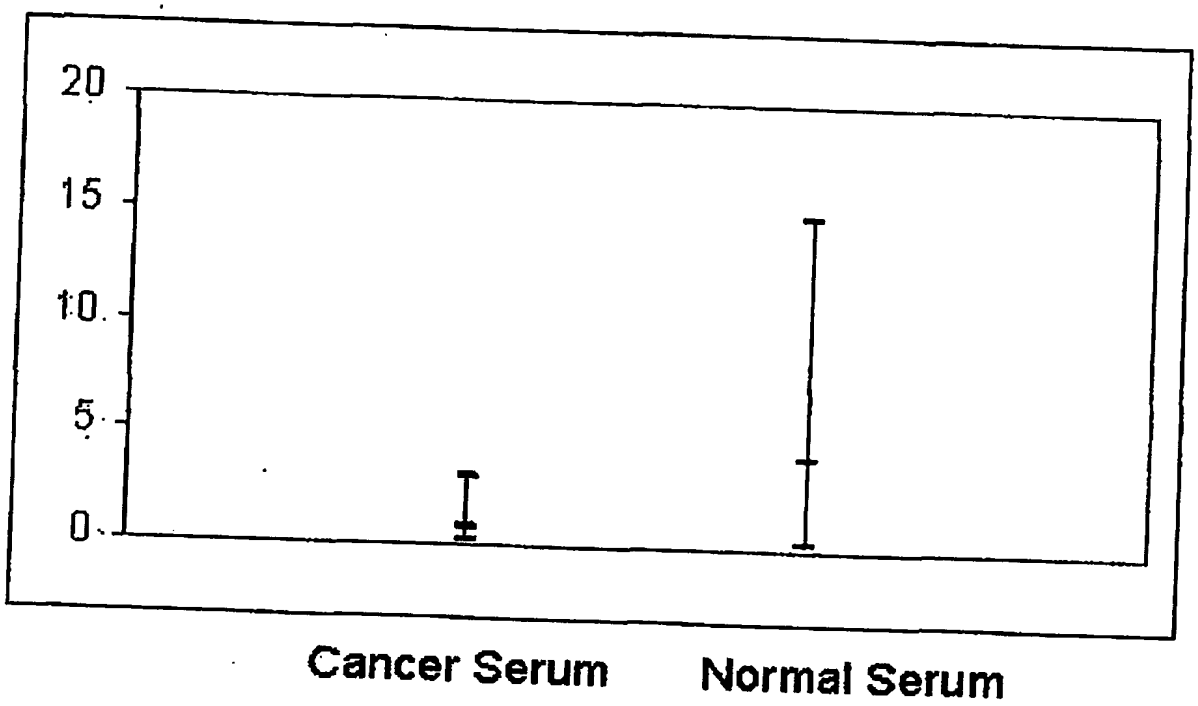

Fig. 8

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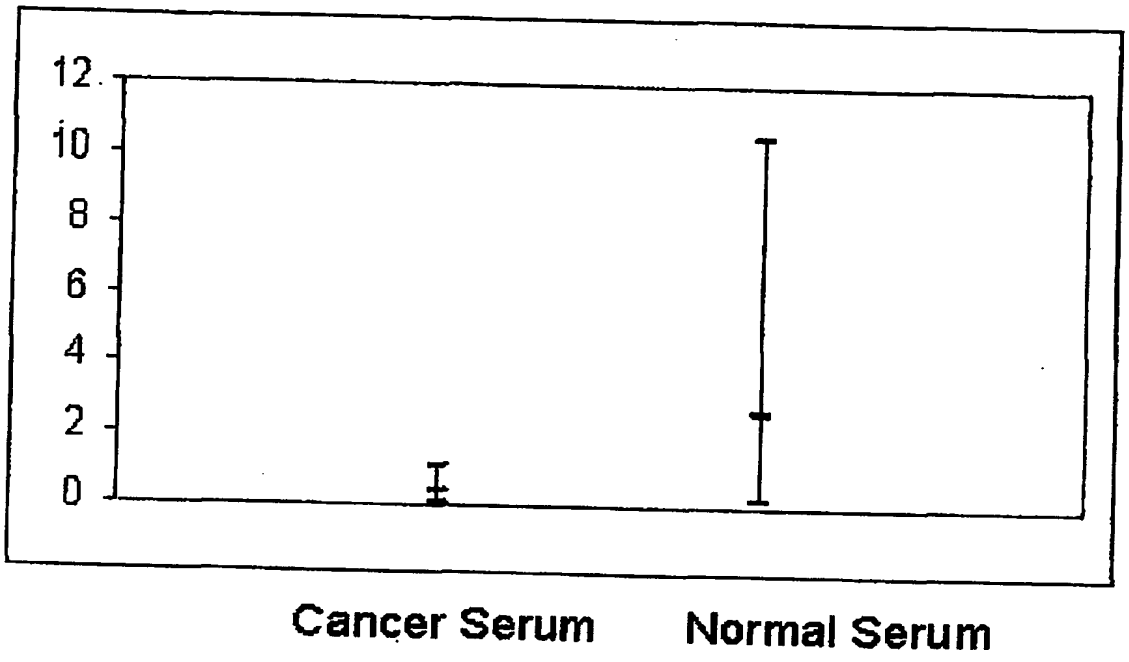

Fig. 9

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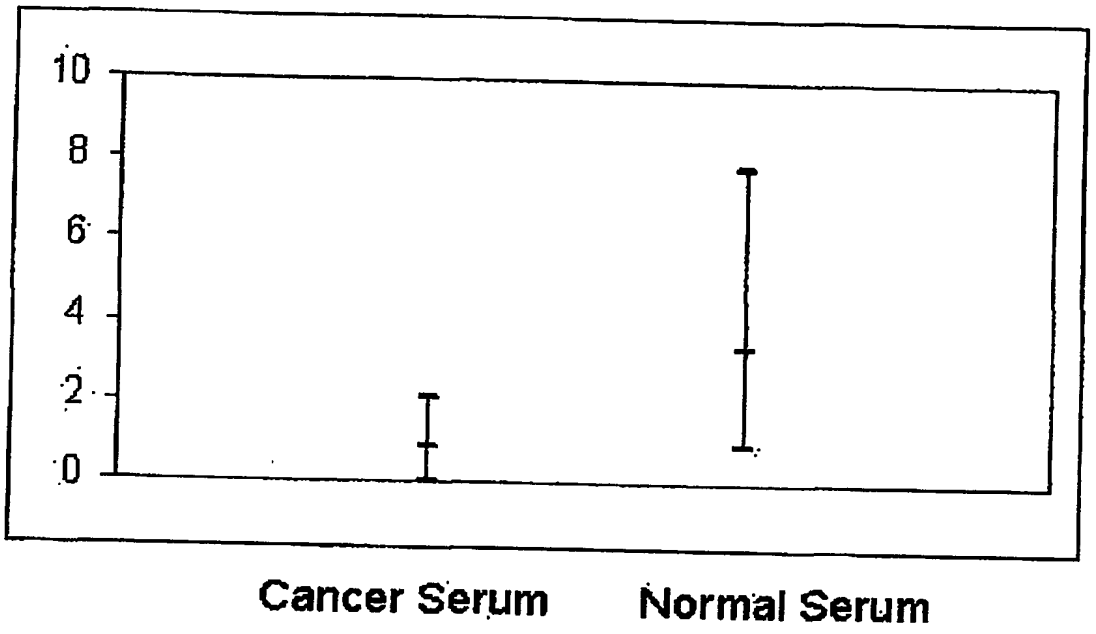

Fig. 10

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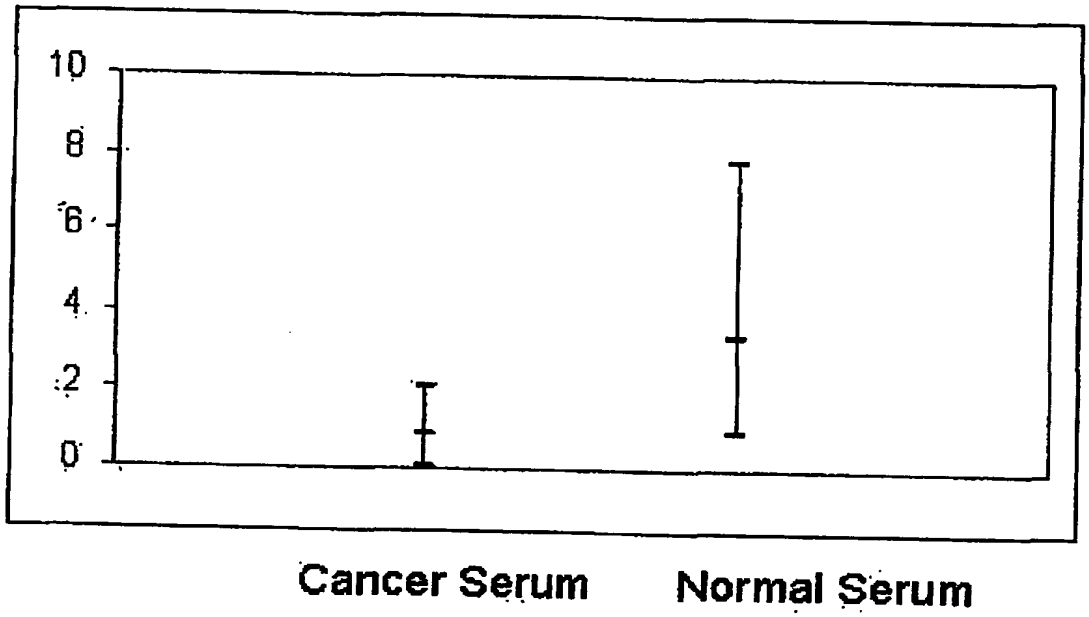

Fig. 11

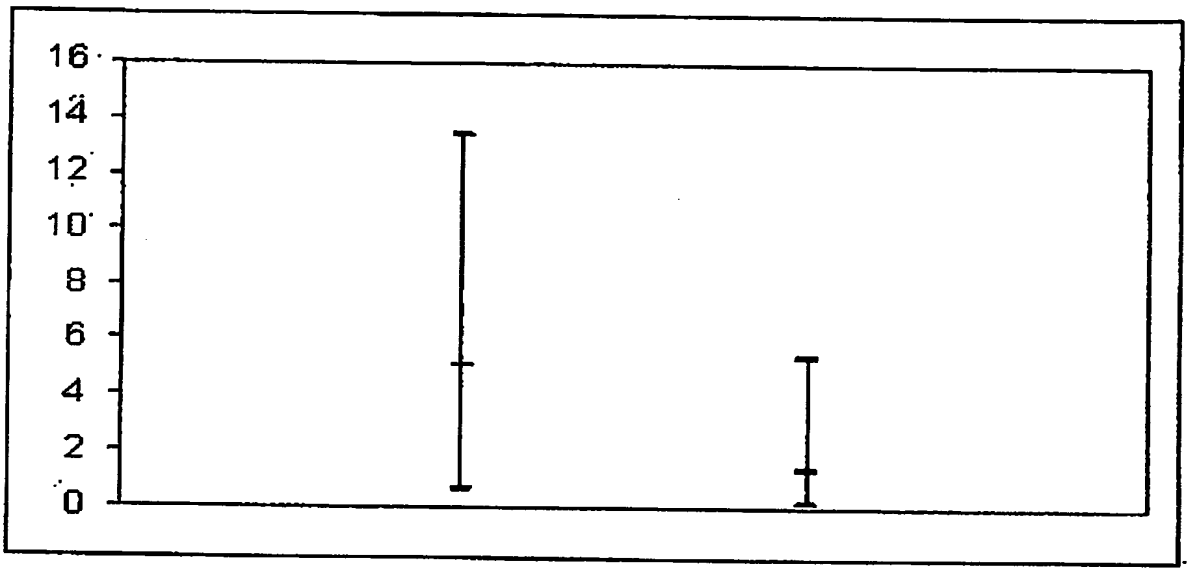

Fig. 12

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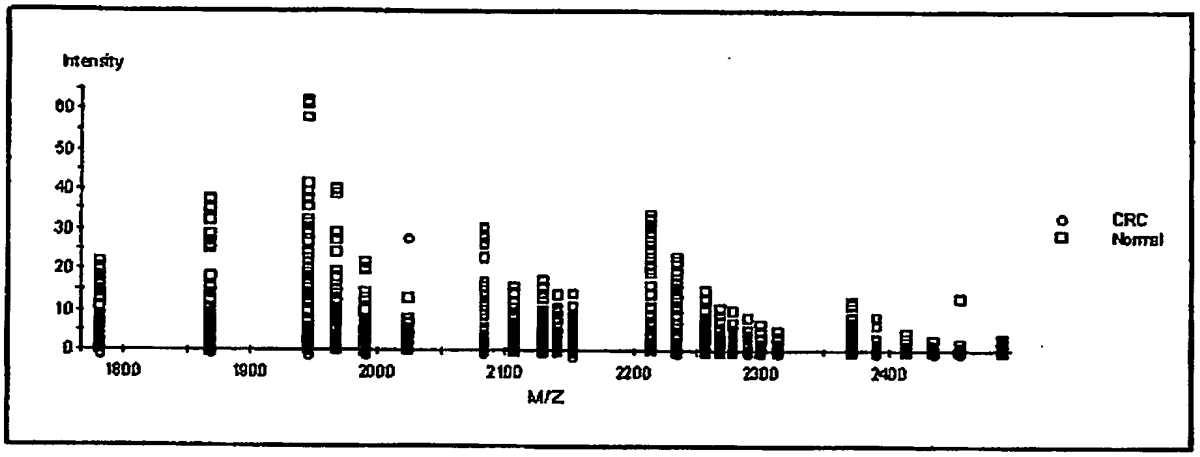

Fig. 13

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